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Phytochelatin-metal(loid) transport into vacuoles shows different substrate preferences in barley and Arabidopsis

Song, Won-Yong ; Mendoza-Cózatl, David G ; Lee, Youngsook ; Schroeder, Julian I ; Ahn, Sang-Nag ; Lee, Hyun-Sook ; Wicker, Thomas ; Martinoia, Enrico

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**Phytochelatin-metal(loid) transport into vacuoles shows
different substrate preferences in barley and Arabidopsis**

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Running Title: Phytochelatin-metal(loid) vacuolar transport in barley

Abstract

Cadmium (Cd) and arsenic (As) are toxic to all living organisms, including plants and humans. In plants, Cd and As are detoxified by phytochelatins (PC) and metal(loid)-chelating peptides and by sequestering PC-metal(loid) complexes in vacuoles. Consistent differences have been observed between As and Cd detoxification. Whereas chelation of Cd by PCs is largely sufficient to detoxify Cd, As-PC complexes must be sequestered into vacuoles to be fully detoxified. It is not clear whether this difference in detoxification pathways is ubiquitous among plants or varies across species. Here, we have conducted a PC transport study using vacuoles isolated from Arabidopsis and barley. Arabidopsis vacuoles accumulated low levels of PC₂-Cd, and vesicles from yeast cells expressing either AtABCC1 or AtABCC2 exhibited negligible PC₂-Cd transport activity compared to PC₂-As. In contrast, barley vacuoles readily accumulated comparable levels of PC₂-Cd and PC₂-As. PC transport in barley vacuoles was inhibited by vanadate, but not by ammonium, suggesting the involvement of ABC-type transporters. Interestingly, barley vacuoles exhibited enhanced PC₂ transport activity when essential metal ions such as Zn(II), Cu(II), and Mn(II) were added to the transport assay, suggesting that PCs might contribute to the homeostasis of essential metals and detoxification of non-essential toxic metal(loid)s.

1 **Keyword Index:**

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3 Phytochelatin transporter, heavy metal, ABC transporter, barley, vacuole, trace metals.

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1 Introduction

2 Metal(loids)s are important raw materials for industry and crucial nutrients for living
3 organisms. Due to their high reactivity, excess metal(lid)s can damage living organisms.
4 Non-essential metal(loid)s, such as As, Cd, Hg, and Pb, can displace essential metals with
5 similar properties; interact with functional groups of catalytic or transport proteins, thereby
6 modifying protein structures; and disturb the cellular redox status by generating reactive
7 oxygen species (Sandalio *et al.* 2001; Sharma and Dietz 2009; Yadav 2010). Many
8 mechanisms to detoxify toxic levels of metal ions in plants have been described, including:
9 inhibition of toxic metal uptake via secretion of chelators, efflux of the metals at the root
10 epidermis, dilution via metal translocation to the shoot, metal chelation in the cytosol, and
11 metal sequestration into vacuoles (Cobbett 2000; Hall 2002; Mendoza-Cózatl *et al.* 2011).
12 Among the molecules that promote heavy metal resistance, phytochelatins (PCs) are the
13 most important in plant systems (Cobbett 2000; Clemens 2006). Phytochelatins are
14 glutathione-derived peptides that tightly bind to heavy metals. First identified as a cadmium-
15 binding peptide (CBP) in *Schizosaccharomyces pombe* (fission yeast; Murasugi *et al.* 1981),
16 these peptides were later also found in plants, algae, and worms (Ahner and Morel, 1995;
17 Grill *et al.* 1987). The importance of PCs in Cd detoxification was elucidated through
18 phenotypic analyses of an *S. pombe* mutant unable to form high molecular mass Cd-
19 phytochelatin-sulfide complexes. Growth of this mutant was severely inhibited compared to
20 its isogenic wild type (Ortiz *et al.* 1991; Speiser *et al.* 1992). After the discovery of PCs,
21 genes encoding phytochelatin synthases (PCS) were identified in *Arabidopsis thaliana*,
22 *Triticum aestivum* (wheat), and fission yeast by Ha *et al.* (1999), Vatamaniuk *et al.* (1999),
23 and Clemens *et al.* (1999), respectively. The *Arabidopsis* *PCS1* knockout mutant is highly
24 sensitive to Cd and As (Ha *et al.* 1999), and heterologous expression of PCS genes from
25 *Triticum aestivum* and *Schizosaccharomyces pombe* in budding yeast enhances Cd
26 resistance (Ha *et al.* 1999). Surprisingly, over-expression of AtPCS1 in *Arabidopsis* does not

enhance tolerance to heavy metals, but rather decreases resistance to Cd and Zn (Lee *et al.* 2003). This result suggests that PCs are essential, but not sufficient, for heavy metal resistance, and that the next step(s), i.e., vacuolar sequestration of PC-metal(loid) conjugates, is required to complete the resistance mediated by phytochelatins.

A putative phytochelatin transporter was first found in *S. pombe*, and named *SpHMT1*. This half-size ABC protein transports PCs as well as PCs conjugated to Cd into the vacuole using energy from ATP hydrolysis (Ortiz *et al.* 1992; Ortiz *et al.* 1995). Salt and Rauser (1995) suggested that plants also have an ABC-type PC transporter at the tonoplast, since oat vacuoles exhibit vanadate-sensitive and ammonium-insensitive PC transport. Recently, Song *et al.* (2010) reported that vacuolar PC-As transport is essential for As resistance, and that AtABCC1 and AtABCC2, which belong to a different ABC clade from SpHMT1, are the major vacuolar PC-As transporters in Arabidopsis (Song *et al.* 2010). The *abcc1abcc2* double mutant is hypersensitive to As and As-containing herbicides, and contains reduced levels of PCs compared to the wild type when treated with As. Furthermore, *AtABCC1* or *AtABCC2* enhance As resistance and PC accumulation in *S. cerevisiae* heterologously expressing *TaPCS1*. Vesicles isolated from yeast expressing either *AtABCC1* or *AtABCC2* together with *TaPCS1* exhibit strong ATP-dependent PC-As transport activity that is sensitive to vanadate and insensitive to ammonium. The same vesicles, however, exhibit low rates of apo-PC₂ transport. Furthermore, *atabcc1 atabcc2* double knockout mutants exhibit negligible vacuolar PC-As transport compared to the high level measured in the wild type.

AtABCC1 and AtABCC2 confer resistance to other heavy metals as well. The *atabcc1* single and *atabcc1 atabcc2* double knockout mutants are hypersensitive to Cd and Hg and over-expression of *AtABCC1* enhances Cd tolerance and accumulation in Arabidopsis (Park *et al.* 2011). However, the Cd sensitive phenotype of the *abcc1 abcc2* double knockout mutant was mild compared to the As or Cd sensitivity of the phytochelatin-deficient *cad1-3*. These results suggest that vacuolar sequestration of PC-Cd might not be strictly required for Cd detoxification in Arabidopsis, but that PC-As sequestration in vacuoles is a key step in As

1 detoxification. Here, we addressed the question of whether the difference in the
2 detoxification pathways for Cd and As can be generalized to other plants. For this purpose,
3 we used barley vacuoles, because they represent an ideal system to study vacuolar
4 transport in monocotyledonous species and are derived from a crop plant. Our results show
5 significant differences in the vacuolar PC-metal(loid) uptake properties between Arabidopsis
6 and barley, suggesting that the dynamics of PC-metal(loid) accumulation in vacuoles varies
7 between plant species.

10 **Materials and Methods**

12 **Plant culture conditions**

13 Barley (*Hordeum vulgare* L. cv. Baraka) was grown in soil in a controlled artificial growth
14 chamber [16/8 h light ($300 \mu\text{E m}^{-2} \text{s}^{-1}$)/dark cycles at 25°C] for 8 days. For experiments
15 using barley vacuoles treated with Cd, barley plants were grown for 5 days in pots containing
16 normal soil, and then treated with 50 μM CdCl_2 solution for 3 additional days. To isolate
17 vacuoles from Arabidopsis, plants were cultured in soil under short day conditions [8/16 h
18 light ($40 \mu\text{E m}^{-2} \text{s}^{-1}$)/dark cycles at 22 °C] for 7- 8 weeks.

20 **PC transport assay in intact vacuoles from barley and Arabidopsis**

21 To isolate vacuoles from barley, protoplasts were released from primary leaves by treatment
22 with MCP buffer (0.5 M sorbitol, 10 mM MES-KOH, pH 5.6, 1 mM CaCl_2) containing 0.6%
23 (w/v) cellulose YC (Seishin Pharmaceuticals, Tokyo) and 0.06% (w/v) pectolyase Y-23
24 (Seishin) for 1 h at 30°C. Protoplasts were collected by centrifugation at 100 g for 7 min on a
25 cushion of Percoll (GE-Healthcare) solution A (Percoll containing 0.5 M sorbitol, 20 mM MES,
26 pH5.6 and 1 mM CaCl_2). To purify mesophyll protoplasts, they were re-suspended in Percoll
27 solution A, and overlaid on 30% (v/v) Percoll solution A diluted with MCP buffer (0.5 M

sorbitol, 20 mM MES, pH 5.6, 1 mM CaCl_2), overlaid with MCP buffer at the top of the tube, and centrifuged at 100 g for 7 min. The intact protoplasts were collected at the interface between the layers of MCP and 30% Percoll solution, mixed with two volumes of lysis buffer (200 mM sorbitol, 10% Ficoll, 20 mM EDTA, 20 mM Hepes-KOH, pH 8, 1.5 mg/ml BSA, and 1 mM dithiothreitol (DTT)), and lysed by incubation in a hot water bath (37-40°C) or mechanical stimulus using a syringe. To purify vacuoles released from the protoplasts, the lysate was overlaid with lysis buffer (without EDTA and DTT) and betaine buffer (400 mM glycine betaine, 30 mM KCl, 20 mM HEPES-KOH, pH 7.2, and 1.5 mg/ml BSA) and centrifuged at 100 g for 7 min. The vacuoles from the top layer were again overlaid with betaine buffer and centrifuged at 100 g for 7 min to remove EDTA and DTT from the vacuoles. The purified vacuoles were resuspended in Percoll-solution [to a final concentration of 10% (v/v) Percoll, 500 mM sorbitol, and 20 mM HEPES-KOH, pH 7.2] and directly used for the PC transport assay.

Two-hundred-and-fifty picomole of PC_2 containing 50 nCi (920 Bq) of ^{35}S - PC_2 per 1 reaction tube was used in the uptake assays. To form PC_2 complexes with As(III), Cd(II), Cu(II), Fe(II), Mn(II), or Zn(II), the metal(loid), PC_2 containing ^{35}S - PC_2 and DTT were mixed at a molar ratio of 1:1:1 and incubated at room temperature for 40 min. Uptake of PCs was assayed using the method described by Martinoia *et al.* (1993). For each condition and time point, four 400 μl polyethylene micro-centrifuge tubes were prepared as follows: 70 μl of transport buffer (22% (v/v) Percoll pH 7.2, 500 mM sorbitol, 30 mM KCl, 20 mM HEPES-KOH, pH 7.2, 0.1% (w/v) BSA) containing 0.05 μCi (920 Bq) $^3\text{H}_2\text{O}$, 2.5 μM PC_2 -metal was combined with 30 μl of vacuole suspension, and then 200 μl silicone oil (AR 200) and 60 μl water were rapidly layered on the mixture. After incubation, vacuoles were floated at the water phase by centrifugation at 10,000 g for 20 s. Radioactivity from the vacuoles was measured using a liquid scintillation counter (Perkin-Elmer). To identify the ratio of Cd/ PC_2 uptake in vacuoles, Cd containing ^{109}Cd and PC_2 containing ^{35}S - PC_2 was incubated at room temperature. Fifty nCi (1840 Bq) of ^{35}S -labelled PC_2 , 50 nCi (1840 Bq) ^{109}Cd , and 50 nCi

(1840 Bq) $^3\text{H}_2\text{O}$ were used as tracers, and 2.5 μM $\text{PC}_2\text{-Cd}$ was used as substrate in each experiment. After the $\text{PC}_2\text{-Cd}$ transport assay, vacuoles were collected and ^{109}Cd radioactivity was detected using a gamma counter (Perkin-Elmer). $^{35}\text{S-PC}_2$ and $^3\text{H}_2\text{O}$ were detected using a liquid scintillation counter. The volume of vacuoles was determined using the value of $^3\text{H}_2\text{O}$.

Cd resistance test in yeast cells

Yeast strains SM4 (*ycf1::His3*, *yhI035c::HIS3-MX6*, *yII015w::Kan-MX6*, *yII048c::TRP1-MX6*) and SM7 (*ycf1::His3*, *yhI035c::HIS3-MX6*, *yII015w::Kan-MX6*, *yII048c::TRP1-MX6*, *TaPCS1::cup1-1*) carrying an empty vector (EV), pNEV-AtABCC1, or pYES3-AtABCC2 were grown in medium at 30°C. To analyse Cd resistance in the yeast lines, yeast cells were cultured in SD ura- medium at 30°C until the mid-log phase (OD600= 2), harvested by centrifugation at 8,000 rpm for 30 s, and then adjusted with water to OD600=10 to 0.01. Three microliters of cells diluted with water were taken from each strain, spotted on the medium with or without CdCl_2 , and cultured at 30°C in an incubator for 3 days.

Isolation of membrane vesicles from yeast and transport assays

Vesicles were prepared from SM7 yeast cells carrying empty vector (*pNEV-Ura*), *pNEV-AtABCC1*, or *pYES3-AtABCC2* grown in SD ura- liquid medium. Before cell wall digestion, cells were incubated in YPD medium for 30 minutes, collected by centrifugation for 5 min at 3000 rpm, and digested with digest buffer (500 mM sorbitol, 25 mM Tris-MES, pH 7.4, 0.5 mM EDTA, 15 mM DTT) containing lyticase (1,000 unit/g fresh weight cells, Sigma) for 0.5 ~ 1 h, and then spheroplasts were harvested by centrifugation for 10 min at 3000 rpm. The cells were washed with 1 M sorbitol, resuspended in homogenization buffer (1.1 M glycerol, 50 mM Tris-ascorbate (pH 7.4), 5 mM EDTA, 1.5% (w/v) polyvinylpyrrolidone(PVP), 1 mM DTT, 2% (w/v) BSA, 1 mM PMSF, and one proteinase inhibitor cocktail tablet (Roche)), and then microsomal vesicles were isolated as described previously (Tomasini et al., 1996).

For the PC₂ transport assay, 50 nCi (920 Bq) of ³⁵S-PCs was used as a tracer of PCs in the PC transport assay. Transport experiments were carried out using transport buffer (4 mM ATP, 5 mM MgCl₂, 10 mM creatine phosphate, 16 units/ml creatine kinase, 1 mg/ml BSA, 100 mM KCl, and 25 mM Tris-MES, pH 7.4) containing 2.5 μM PC₂ for the indicated times. Before starting the PC transport experiments, 10 μl of vesicles (100 μg of protein) was mixed with 90 μl of transport buffer containing substrate and kept on ice for 5 minutes. The reactions were performed at 25°C, stopped by adding 1 ml of ice-cold washing buffer (100 mM KCl and 25 mM Tris-Mes (pH 7.4)), filtered immediately under vacuum through a 0.45-μm-diameter pore size nitrocellulose filter (Millipore), and washed two times with 2 ml of washing buffer. Vanadate (1 mM) and NH₄Cl (5 mM) were used as an inhibitor of ATPase and pH gradient, respectively.

³⁵S-phytochelatin-2 synthesis

³⁵S-PC₂ was synthesized using recombinant AtPCS1-6xHis protein and ³⁵S-GSH HPLC, as previously described (Song et al., 2010). ³⁵S-GSH was purchased from PerkinElmer (MA, USA) and the specific activities ranged from 660 to 840 Ci/mmol between batches. PCs were separated by HPLC, monitored at 220 nm, and confirmed by mass spectrometry, as previously described (Mendoza-Cózatl et al., 2008). ³⁵S-PC_s were finally re-suspended in 0.1 M Tris (pH 8.0), 0.5 mM DTT, and 0.5 mM of non-radioactive PC₂ (AnaSpec, CA, USA). After purification, the specific activity of ³⁵S-PC₂ varied between 2 and 5.9 nCi/mmol.

Phylogenetic analysis of the plant ABCC1 family

All ABCC protein sequences of Arabidopsis, rice, Brachypodium, maize, and grape were collected from the ARAMEMNON website (<http://aramemnon.botanik.uni-koeln.de/>), and a barley AtABCC1 ortholog was identified through the blastp program in the Barley BLAST Server (<http://webblast.ipk-gatersleben.de/barley/viroblast.php>).

All members of the plant ABCC protein family were aligned using ClustalW programs with

default values in MEGA and BioEdit software, and phylogenetic analyses were conducted using MEGA version 4 (Tamura et al., 2007) using amino acid sequence homologies. A phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei, 1987). The bootstrap values (percentage) of 1000 replicates are shown at the branching points.

Results

PC₂-Cd transport in *Arabidopsis thaliana*

In two former studies, it was shown that AtABCC1 and AtABCC2 play an important role in arsenic (As) and cadmium (Cd) resistance (Song et al., 2010; Park et al., 2011). To compare the detoxification efficiency of As and Cd through vacuolar sequestration of PC-Cd and PC-As mediated by AtABCC1 and AtABCC2 in *Arabidopsis*, we compared the sensitivities of the *atabcc1 atabcc2* double mutant and the PC-deficient mutant *cad1-3* to As and Cd. As summarized in Fig. 1, *atabcc1 atabcc2* double knockout plants were not as sensitive to Cd as was *cad1-3*. Increasing the Cd concentration from 20 to 40 μ M severely limited growth of the *atabcc1 atabcc2* mutant, while growth was almost completely abolished in the *cad1-3* mutant at 30 μ M (Fig. 1a, c). In contrast, *atabcc1 atabcc2* and *cad1-3* mutants exhibited similar and almost complete root growth inhibition (90%) in medium containing 20 μ M As (V). In both mutants, growth was completely arrested on plates containing 30 μ M As(V) (Fig. 1b, d). These results indicate that chelation of Cd by PCs is sufficient to detoxify the majority of cytosolic Cd, but that vacuolar sequestration by AtABCC1 or AtABCC2 is necessary for full detoxification of As.

Since vacuolar sequestration of PC-Cd might not be strictly required for efficient detoxification of Cd in *abcc1 abcc2* (Fig. 1a-d), we tested whether the major PC₂-As transporters, AtABCC1 and AtABCC2, can also efficiently sequester Cd conjugated with PCs (PC₂-Cd) *in vitro*. The transport activity of PC₂-Cd was 1.7 pmol/1 μ l vacuole/ 15 min (Fig. 1e). Transport was abolished by vanadate, an inhibitor of P-type ATPases and ABC transporters, but not by NH₄Cl, which dissipates the proton gradient (Fig. 1e). Similar results

were described for PC₂-As transport mediated by AtABCC1 and AtABCC2, as reported by Song *et al.* (2010).

To test whether AtABCC1 and AtABCC2 confer cadmium resistance by compartmentalizing a GS-Cd or PCs-Cd complex into the vacuole, we expressed the transporters in SM4, a quadruple mutant of four vacuolar ABCC-type ABC transporters, and SM7, a yeast mutant expressing the wheat PC synthase1 in the SM4 background. Surprisingly, neither *AtABCC1* nor *AtABCC2* expression increased Cd tolerance in yeast, and *AtABCC2* expression increased Cd sensitivity in both yeast strains, regardless of whether or not the transporters were co-expressed with a wheat phytochelatin synthase1 gene (Fig. 2a). Nonetheless, vesicles from yeast expressing *AtABCC1* and *AtABCC2* exhibited PC₂-Cd transport activity. This activity was much lower than that observed for PC₂-As (Fig. 2b). For instance, PC₂-Cd transport activities mediated by AtABCC1 and AtABCC2 were only 1.52- and 1.47 times higher than those of the empty vector control, which is significantly lower than the >500-fold increase in PC₂-As transport activity observed in vesicles expressing either *AtABCC1* or *AtABCC2* (Fig. 2b). These results suggest that AtABCC1 and AtABCC2 can transport PC₂-Cd, but have a strong preference for PC₂-As as their substrate.

PC uptake in barley vacuoles is activated by cadmium

To obtain more biochemical information about phytochelatin-Cd transport activity in other plant species, and to establish whether PC transport is similar in two different plant systems, we carried out PC-Cd transport assays using barley vacuoles isolated from barley mesophyll protoplasts. First, we examined the transport activities of barley vacuoles for PC₂-Cd and PC₃-Cd complexes. In the absence of Mg-ATP, there was no transport of PC₂-Cd or PC₃-Cd, and the transport activities of apo-PC₂ and apo-PC₃ were extremely low, even in the presence of Mg-ATP (Fig. 3a). However, the transport activities for PC₂ and PC₃ were enhanced in the presence of Cd. PC₂ and PC₃ transport into vacuoles was 53 times and 17

times greater when Cd was present in the uptake assay. Uptake of PC₂-Cd into vacuoles was time- and ATP-dependent (Fig. 3b). To determine whether Cd acts only as an activator of PC transport or whether Cd conjugated with PC₂ is actually transported into vacuoles, we analyzed PC₂ and Cd concentrations in vacuoles incubated in the presence of PC₂-Cd with or without Mg-ATP for 15 minutes. To produce the PC₂-Cd complex, we mixed PC₂ and CdCl₂ at a one-to-one molar ratio, and performed a double tracer experiment using ³⁵S-PC₂ and ¹⁰⁹Cd as tracers for PC₂ and Cd, respectively. In the presence of Mg-ATP, uptake of PC₂ and Cd increased by 80% and 100% compared to assays in which no Mg-ATP was added (Fig. 3c). Therefore, the molar ratio of PC₂ to Cd transported by Mg-ATP was close to one. A similar ratio was reported for PC transport assays performed by Salt and Rauser (1995). These authors found that PC₃, which contains three thiols per molecule, and Cd were transported at ratios of 1:1.7 and 1:1.5 (PC₃:Cd), respectively, when measured as vanadate-sensitive or NH₄Cl-insensitive transport.

To further characterize the vacuolar PC₂ transport characteristics in barley vacuoles, we performed PC₂ transport assays under different conditions (Fig. 3d). As expected, when the transport reaction was performed on ice, no PC₂-Cd transport activity was observed. Apo-PC₂ transport activities were not influenced by reducing and oxidizing agents (1 mM DTT and 1 mM GSSG, respectively, Fig. 3d). As observed for Arabidopsis vacuoles, PC₂-Cd transport activity was strongly inhibited by vanadate, but not affected by NH₄Cl treatment. These results indicate that ATP hydrolysis is required for vacuolar PC-Cd transport, but that the proton gradient is not. Finally, we compared PC₂-Cd and PC₂-As transport activities in vacuoles isolated from barley leaves. In contrast to what was observed in Arabidopsis, PC₂-Cd transport activity was as high as PC₂-As uptake (Fig. 3e). This result indicates that, although both plants can efficiently transport PCs, the affinities for the different complexes may vary between plant species.

Phytochelatin may essential metals in barley vacuoles

Plants often take up excess amounts of essential heavy metals. To test whether these metals can also be transported into vacuoles as PC conjugates, we produced PC₂ complexes with several metal ions by incubating PC₂ and the ions (at one-to-one ratios) for one hour at room temperature. We added the PC₂ complexes into the transport buffer containing Mg-ATP. The transport activities observed for PC₂ complexes with As(III), Mn(II), Cu(II), and Zn(II) were much higher than those detected for apo-PC₂ (Fig. 4a), and similar to those observed for the PC₂-Cd complex. In contrast, the uptake of the PC₂-Fe complex was not significantly higher than that of apo-PC₂. Furthermore, the transport activities of PC₂-Mn, PC₂-Cu, and PC₂-Cd were consistently higher in vacuoles isolated from barley treated with 100 µM Cd(II) for 3 days compared to vacuoles isolated from untreated control plants (Fig. 4b). This suggests that the transport activity of PC₂-metal complexes was induced by Cd treatment. These results also indicate that essential metal ions can be transported into vacuoles via the PC-dependent pathway, and that this pathway can be activated by exposure to non-essential metals like Cd.

Putative phytochelatin transporters in monocots

To explore whether monocotyledonous plants have AtABCC1 and AtABCC2 orthologs encoding putative PC₂-Cd transporters, we developed a phylogenetic tree of ABCC-type transporters in monocot and eudicot plants based on amino acid sequence alignments (Fig. 5). In this analysis, we included *Hordeum vulgare* and *Sorghum bicolor* putative orthologs of ABCC1, ABCC2, and ABCC-type transporters from *Arabidopsis thaliana*, *Populus trichocarpa*, *Vitis vinifera*, *Oryza sativa*, *Zea mays*, and *Brachypodium distachyon*. Using the blastp program in the Barley BLAST Server, we identified only two partial sequences of an AtABCC1 barley ortholog, i.e., MLOC_15955 and MLOC_56261. Whereas MLOC_15955 covers approximately 583 amino acids of the N-terminus, MLOC_56261 covers 965 amino acids at the C-terminus. However, a segment covering approximately 90 amino acids between the two termini is not represented in the publicly available genome sequence, as

the recently published barley genome sequence (International Barley Genome Sequencing Consortium, 2012) is incomplete. The protein sequence of the barley putative ortholog (Hvul_ABCC1) is provided as a supplementary file (Fig S1).

Phylogenetic analysis of the plant ABCC family revealed that the PC₂-As transporters AtABCC1 (At1g30400) and AtABCC2 (At2g34660) form a subgroup distinct from other ABCCs (Fig. 5a). Included in the same subgroup with AtABCC1 and AtABCC2 were ABCC12 (At1g30410) and ABCC11 (At1g30420) of Arabidopsis, ABCC1 (Os04g52900) of rice, three members of poplar ABCCs, one member of Brachypodium, and one member each of grape, maize, sorghum, and barley (Fig. 5a green circle). ABCC-type transporters OsABCC1 (Os04g52900), Bd5g22080, Zm084181, Hvul_ABCC1, and Sb06g028880 from rice, Brachypodium, maize, barley, and sorghum, respectively, were closely related to the major Arabidopsis PC₂-As transporters AtABCC1 and AtABCC2 (Fig. 5a, red circle). However, three ABCC homologs in poplar (Pt0004s03470, Pt0249s00200, and Pt0011s04290) and grape (Vv0028393001) were more similar to Arabidopsis ABCC12 (At1g30410) and ABCC11 (At1g30420), whose knockout mutants did not exhibit any arsenical pesticide disodium methanearsonate (DSMA) phenotype (Song et al., 2010). Interestingly, phylogenetic analysis showed that the four closest grass homologs cluster together in an order that reflects the phylogenetic relationships of the four species (i.e., barley, rice, sorghum, and Brachypodium) and that each grass contains only one member of this clade. Arabidopsis ABCC1 and 2 exhibited a high level of amino acid sequence identity with the monocot AtABCC1 and 2 putative orthologs; 71.2% with OsABCC1 of *Oryza sativa*, 72.4% with Zm084181 of *Zea mays*, and 70.6% with Bd5g22080 of *Brachypodium distachyon*. AtABCC1 and AtABCC2 orthologs in monocots had a high level of amino acid sequence identity, although *Oryza sativa*, *Sorghum bicolor*, and *Brachypodium distachyon* belonged to different subfamilies. OsABCC1 and Sb06g028880, OsABCC1 and Bd5g22080, and Sb06g028880 and BdABCC1 shared 91.2%, 92.1% and 90.8% amino acid identity, respectively.

Although the ABCC1 and ABCC2 orthologs in monocots and dicots have high levels of amino acid sequence identity, there is a distinct sequence proximal to amino acid position 842 - 879 in AtABCC1 between ABCC domain and the second ABC membrane domain, where AtABCC1 and 2 and their monocot orthologs exhibited only 22% ~ 35% amino acid identity. However, ABCC1 monocot orthologs had a high amino acid identity of 59%~78% at this position (Fig. 5b). Therefore, ABCCs of monocots and Arabidopsis cluster to distinct groups in this phylogenetic analysis (Fig. 5b).

Discussion

Cd resistance mediated by ABCC transporters

PCs were identified as being responsible for the major detoxification mechanism of a large number of metal(loid)s in plants, *S. pombe*, and *C. elegans* (Clemens *et al.* 2001; Grill *et al.* 1987; Murasugi *et al.* 1981). When PCS1 was deleted or mutated, all these organisms exhibited severe growth inhibition when grown on media containing Cd, As, or Hg (Clemens *et al.*, 1999; Ha *et al.* 1999; Vatamanuik *et al.* 2001). PC synthase is constitutively expressed, but PC synthesis is only induced in the presence of non-essential metals, such as Cd(II), As(III), and Hg(I), or an elevated concentration of essential transition metals, such as Zn(II) and Cu(II) (Ahner and Morel 1995; Grill *et al.* 1989; Vatamanuik *et al.* 2001). It is widely accepted that PCs form complexes with metal(loid)s and that these complexes are transported into the vacuole for final detoxification. However, due to the different stability constants of metal(loid)-PC complexes, which are further affected by pH, the requirements for the efficient transport of PC conjugates into vacuoles may differ between metal(loids) and thus be reflected by different transport rates (Fig. 4).

The *S. pombe* mutant *hmt1* was unable to form high molecular weight PC-Cd (HMWPC-Cd-S-2) complexes (Ortiz *et al.*, 1992; Ortiz *et al.*, 1995). This mutant was hypersensitive to Cd, but not to As or Hg. In contrast, the PC-deficient mutant *pcs* was hypersensitive to As, Hg, and Cd. SpHMT1 has been reported to transport apo-PC and PC-Cd. Recent results

1 indicate that Cd detoxification mediated by HMT1 and its orthologs may result from a
2 mechanism other than vacuolar transport of PCs. For instance, DmHMT-1, a SpHMT1
3 ortholog in *D. melanogaster*, which does not produce PCs, could not complement vacuolar
4 PC sequestration of the *hmt1* pombe mutant, although it could rescue the Cd
5 hypersensitivity of *hmt1* (Sooksa-Nguan *et al.* 2009). Moreover, SpHMT1 enhanced the Cd
6 resistance of *S. cerevisiae* and *E. coli*, which do not produce PCs (Preveral *et al.*, 2009).
7 These results suggest that SpHMT1 may transport Cd bound to different ligands, including
8 but not limited to PCs. The first direct demonstration that full-length ABCC-type transporters
9 mediate PC transport into yeast and plant vacuoles was published in 2010 (Mendoza-Cózatl
10 *et al.* 2010; Song *et al.* 2010). While *S. pombe* ABCCs were suggested to mediate Cd
11 detoxification, two plant ABC transporters, AtABCC1 and AtABCC2, were shown to
12 sequester PC-As into vacuoles (Mendoza-Cózatl *et al.* 2010; Song *et al.* 2010). Later studies
13 showed that AtABCC1 and AtABCC2 also confer Cd tolerance in Arabidopsis (Park *et al.*
14 2011). Using fluorescent dyes to report the intracellular localization of Cd, Park *et al.* (2012)
15 showed that Cd predominantly localized to the vacuoles of wild-type plants, whereas it was
16 almost exclusively found in the cytosol of *abcc1abcc2* double mutants. Notably, in contrast to
17 the arsenic sensitivity of *abcc1 abcc2* was comparable to plants lacking PCs, the *abcc1*
18 *abcc2* had a less impact on Cd sensitivity compared to the PC-deficient mutant (Park *et al.*
19 2011; Fig. 1a). Because PC-Cd transport was not addressed in that study, we were
20 interested in comparing the transport activities of PC-As and PC-Cd complexes. Surprisingly,
21 AtABCC1 and 2 did not enhance Cd resistance. SM4 and SM7 cells expressing AtABCC1
22 were even more sensitive to Cd than was the empty vector control. The cadmium
23 hypersensitivity mediated by AtABCC21 overexpression might be caused by decreased
24 levels of GSH or PCs in the cytosol, because AtABCC21 could transport GSSG, GS
25 conjugates, and apoPCs into the vacuole (Lu *et al.*, 1998; Song *et al.*, 2010). Vacuoles
26 isolated from Arabidopsis also exhibited low levels of PC₂-Cd transport activity. In contrast,
27 we found high PC₂-As transport activity in yeast and plants expressing AtABCC1 and 2 (Fig.

1 1 and 2, Song *et al.*, 2010). Barley vacuoles, on the other hand, exhibited similar transport
2 rates for PC₂-Cd and PC₂-As (Fig. 3). The transport activity was dependent on Mg-ATP, and
3 inhibited by vanadate, but not by ammonium (Fig. 3d). These results imply that barley has a
4 vacuolar ABC transporter for PC complexes which differs in its substrate specificity from
5 those described from Arabidopsis (Song *et al.* 2010).

6 According to the amino acid sequence analysis of the ABCC subfamily members in
7 plants, the orthologs of AtABCC1 and AtABCC2 form a distinct group of ABCC transporters
8 (Fig. 5). This suggests an evolutionary relationship with respect to the detoxification of toxic
9 metal(loid)s through vacuolar compartmentation of PC-metal. Interestingly, monocot ABCC
10 proteins belonged to the same clade as Arabidopsis ABCC1 and ABCC2, whereas ABCC
11 proteins of the tree plants *V. venifera* and *P. trichocarpa*, which have high levels of homology
12 with AtABCC1 and AtABCC2, belonged to the ABCC12 (At1g30410) and ABCC11
13 (At1g30420) subclades, respectively. Arabidopsis *abcc11* and *abcc12* were not sensitive to
14 As or arsenic-containing pesticides (Song *et al.*, 2010). Monocot AtABCC1 orthologs have
15 70 ~ 72% identity with AtABCC1, but the region between the first ABCC domain 1 (ATP
16 binding site) and the second ABC membrane domain (from amino acids 842 to 889) has only
17 22% ~ 35% amino acid sequence identity with AtABCC1. Monocot proteins have high levels
18 of amino acid sequence identity (59%~78%) in this region. We suggest that the regions
19 might be responsible for the different PC-Cd transport functions in monocot and dicot
20 ABCCs.

22 **The role of vacuolar PC transport and nutrient homeostasis**

23 In addition to the detoxification of non-essential metal(loid)s, PCs have also been suggested
24 to mediate the detoxification of essential metals when present in excess. This has been
25 discussed based on the fact that PCS is constitutively expressed and several heavy metals
26 may at least slightly induce PC synthesis. Furthermore, during heavy metal stress, PCs may
27 also form complexes with essential nutrients. Since these essential transition metal ions,

1 such as Zn(II), Cu(II), and Mn(II), have similar chemical characteristics as some non-
2 essential toxic metals ions, such as Cd(II) or Hg(I), they may bind with these thiol-
3 containing compounds (Goyer 1997; Yadav 2010). To test whether essential metals
4 conjugated with PCs can be sequestered into the vacuole, we performed PC₂-metal
5 transport assays using barley vacuoles. Surprisingly, barley vacuoles transported Cu(II),
6 Zn(II), and Mn(II) conjugated with PC₂ in the presence of Mg-ATP (Fig. 4A), and the
7 transport activities were slightly higher in vacuoles isolated from barley plants grown in soil
8 supplemented with Cd for 5 days than in those grown in uncontaminated soil (Fig. 4b). Thus,
9 we suggest that essential metal ions, such as Zn(II), Cu(II), and Mn(II), can be transported
10 into vacuoles as forms of PC₂-metal complexes through the putative ABC transporter(s). In
11 accordance with this explanation, there are direct and indirect lines of evidence that suggest
12 that PCs are involved in the regulation of transition metal ion homeostasis. For example, PC
13 synthesis is activated by essential metal ions as well as non-essential metal ions under both
14 *in vivo* (Ahner and Morel 1995; Grill *et al.* 1987; Maitani *et al.* 1996) and *in vitro* conditions
15 (Vatamanuik *et al.* 2000). In addition, the PC-Cu complex is present in plant cells treated
16 with Cu (Grill *et al.* 1987; Maitani *et al.* 1996). Finally, *cad1-3* and *sppcs1*, *PCS1* defective
17 mutants of *A. thaliana* and *S. pombe*, respectively, exhibited Cu hypersensitivity (Clemens *et*
18 *al.* 1999; Ha *et al.* 1999). Similarly, *cad1-3* and *cad1-6* were more sensitive than the WT
19 and accumulated less Zn when grown on low-strength cation medium (one tenth Hoagland
20 medium) supplemented with 50 μ M Zn (Tennstedt *et al.* 2009), suggesting that PCs can
21 regulate cytosolic zinc ion levels by forming a complex with Zn.

22 In conclusion, we have demonstrated that barley has a vacuolar transporter for PC-Cd,
23 and that an ABC-type transporter may participate in the vacuolar PC-Cd transport by
24 detoxifying excess Cd. This discovery advances our understanding of metal(loid)
25 detoxification mechanisms in plants other than Arabidopsis. In addition, it is still unclear
26 whether vacuolar sequestration of PC-Cd complexes is indeed the final step for Cd
27 detoxification, although many studies have shown that PCs are the major Cd-binding

1 peptides for Cd detoxification in plants, yeast, and nematodes (Clemens *et al.* 1999; Ha *et al.*
2 1999; Vatamaniuk *et al.* 1999; Vatamanuik *et al.* 2001). Our phylogenetic tree indicates that
3 some putative candidates may act as vacuolar PC transporters in barley and monocots.
4 Verification of their transport activity and further investigation of their role in conferring
5 essential heavy metal resistance will be a fascinating topic for future research.

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Figure legends

Figure 1. PC-deficient and PC transporter mutants show differential sensitivity to As and Cd. (a-d) Phenotypic analysis of Cd and As toxicity in Arabidopsis *ABCC1* and 2 double knockout (*abcc1 abcc2*) and phytochelatin synthase (*cad1-3*) mutants. Seeds of *atabcc1,2*, *cad1-3*, and Col-0 (WT) were sown on half-strength MS agar medium supplemented with or without CdCl₂ (a) or NaHAsO₄·7H₂O (b) and grown for 12 days before the root lengths were measured (c, d) (n=10, N=2). (e) PC₂-Cd transport activity in vacuoles isolated from Arabidopsis mesophyll protoplasts. The transport activity of apo-PC₂ and PC₂-Cd (final concentrations of 2.5 μM in the buffer) was assayed in the presence or absence of 5 mM NH₄Cl or 1 mM vanadate. The PC₂ content was measured after an incubation time of 15 min at room temperature. The values indicate the averages and standard errors of values obtained in the absence of Mg-ATP subtracted from those obtained in the presence of Mg-ATP (n=4, N=2).

Figure 2. Vacuolar sequestration of PC₂-Cd mediated by AtABCC1 and AtABCC2 in yeast mutant lines. (a) Phenotypic analysis of yeast lines SM4 and SM7 expressing *AtABCC1* or *AtABCC2* in the presence or absence of various concentrations of Cd. Yeast cells harboring empty vector, *AtABCC1*, or *AtABCC2* were subcultured in SD ura- liquid medium, spotted on SD ura- agar plates supplemented with or without CdCl₂, and cultured in a 30°C incubator for 3 – 4 days. (b) Transport activities for apo-PC₂, PC₂-Cd, and PC₂-As in yeast vesicles isolated from yeast cells expressing *AtABCC1*, *AtABCC2*, or empty vector (EV). Apo-PC₂,

PC₂-Cd, and PC₂-As (final concentrations of 2.5 μ M in the assay buffer) were added to transport assay buffer supplemented with or without Mg-ATP, and the reactions were incubated at room temperature for 40 min. The values indicate the average and standard error obtained in the absence of Mg-ATP subtracted from those obtained in the presence of Mg-ATP (n=4, N=2). Significant differences were calculated relative to the corresponding EV control value. **P* < 0.05, ***P* < 0.01 (Student's *t*-test).

Figure 3. Figure 3. PC₂-Cd transport assay in vacuoles isolated from barley mesophyll cells. (a) ATP-dependent PC₂ and PC₃ transport assay. Apo-PC₂, apo-PC₃, PC₂-Cd and PC₃-Cd (final concentrations of 150 μ M in the assay buffer) were added to transport buffer containing purified barley vacuoles, and the reactions were incubated at room temperature for 2.5 and 17.5 min, respectively. The values were calculated by deducing the 2.5 min from the 17.5 min values to remove the non-specific adsorption. Values represent averages and standard errors (n = 5). (b) Time-dependent PC₂-Cd transport assay. The final concentration of PC₂-Cd was 2.5 μ M in this transport assay. The values represent averages and standard errors of values obtained at the indicated time points minus those obtained after 2.5 min of incubation (n = 4, N = 2). (c) PC₂ and Cd concentrations in barley vacuoles after an incubation time of 15 min in the absence (–) or presence (+) of Mg-ATP. PC₂-Cd was added (final concentration of 2.5 μ M in the assay buffer). ³⁵S-PC₂ and ¹⁰⁹Cd were used as tracers for PC₂ and Cd in this study (n = 4, N = 2). (d) Effects of inhibitors of PC₂-Cd uptake into barley vacuoles. Barley vacuoles were incubated in transport buffer supplemented with 1 mM DTT, 1 mM GSSG, 5 mM NH₄Cl or 1 mM vanadate, respectively for 15 min. Values indicate averages and standard errors (n = 4, N = 2). (e) Comparison of PC₂-Cd and PC₂-As transport activities. Barley vacuoles were incubated in transport buffer supplemented with or without Mg-ATP for 15 min. The values indicate averages and standard errors (n = 4, N = 2). The values in (d) and (e) represent values obtained in the absence of Mg-ATP subtracted from those obtained in the presence of Mg-ATP.

Figure 4. Essential metal ions increase PC transport activity in barley vacuoles. (a) Transport activity of PC₂ complexes in the presence of essential metal ions. (b) Comparison of PC₂ transport activity in barley vacuoles isolated from plants grown on soil with or without 50 μ M CdCl₂. Apo-PC₂ and PC₂ complexes (one-to-one ratio of PC₂ and metal ion) with the indicated metal ions were added to transport buffer containing vacuoles. The final concentrations of apo-PC₂ and PC₂ complexes in the transport medium were 2.5 μ M, and the reactions were incubated at room temperature for 15 min. The values indicate averages and standard errors from values obtained in the absence of Mg-ATP subtracted from those obtained in the presence of Mg-ATP (n=4, N=2). **P* < 0.05, ***P* < 0.01 (Student's *t*-test).

Figure 5. Phylogenetic analysis of genes similar to AtABCC1 in plants. (a) Phylogenetic tree of genes related to AtABCC1 in plants. Green circle: sub-group of AtABCC1 and 2; red circle: AtABCC1 and 2 orthologs). The phylogenetic tree was generated from multiple alignments of ABCC protein sequences. (b) Comparison of amino acid sequences of AtABCC1 and 2 and their putative monocot orthologs. The upper figure indicates a distinct region (amino acid positions 842 to 889) of AtABCC1, 2, and their monocot orthologs. The middle figure compares the amino acid sequences from a distinct region of AtABCC1, 2, and their monocot orthologs. The bottom figures show the phylogenetic tree generated by multiple alignments of amino acid sequences from the distinct region of AtABCC1, 2, and their monocot orthologs indicated in the middle figure. BioEdit software was used for this alignment. Identical amino acid residues are in black boxes.

Phylogenetic analyses were performed using MEGA version 4.0 and were based on multiple alignments of ABCC protein sequences generated by the neighbor-joining method. The bootstrap values (percentage) of 1000 replicates are shown at the branching points. The scale bars indicate unit of branch length.

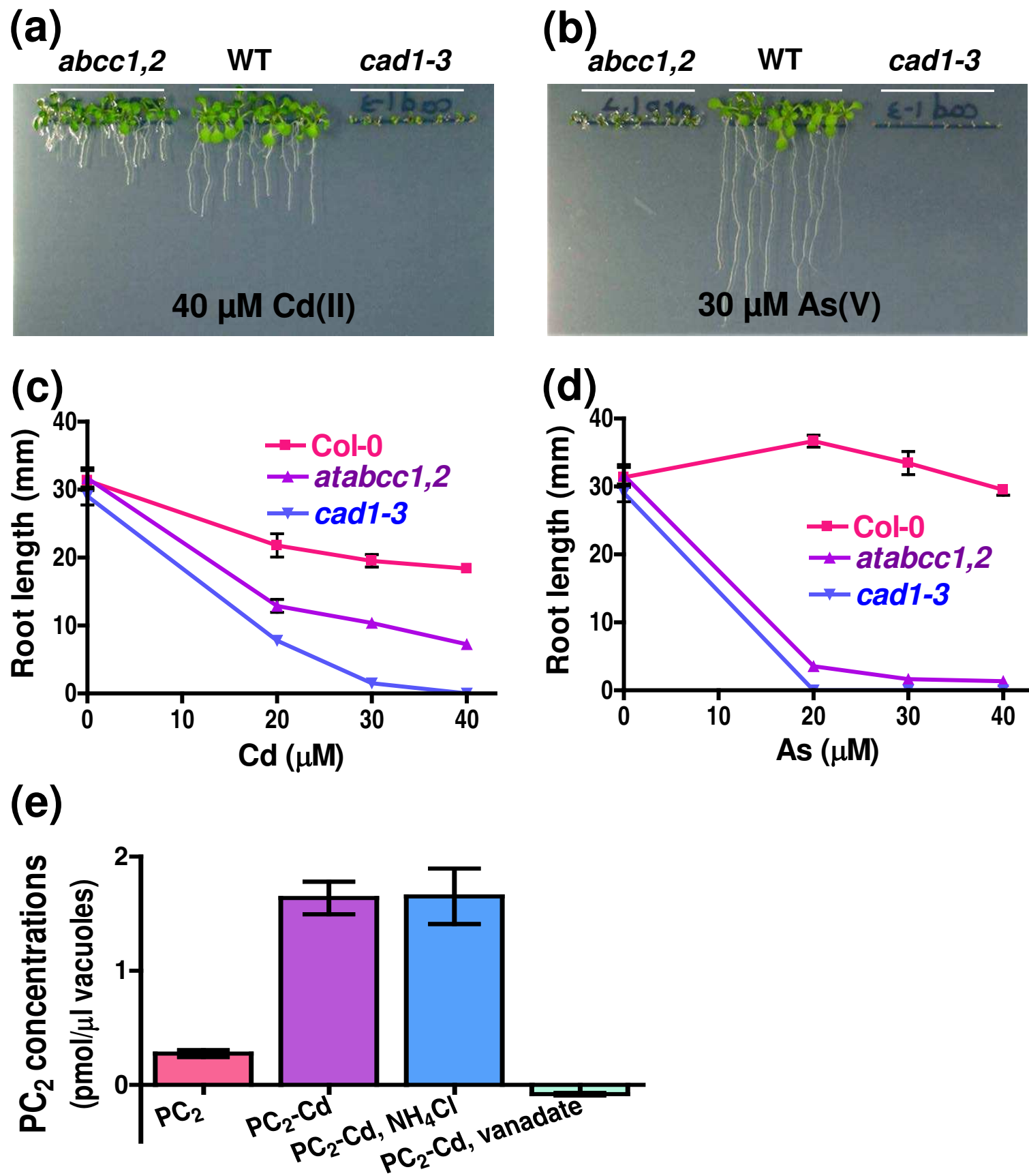
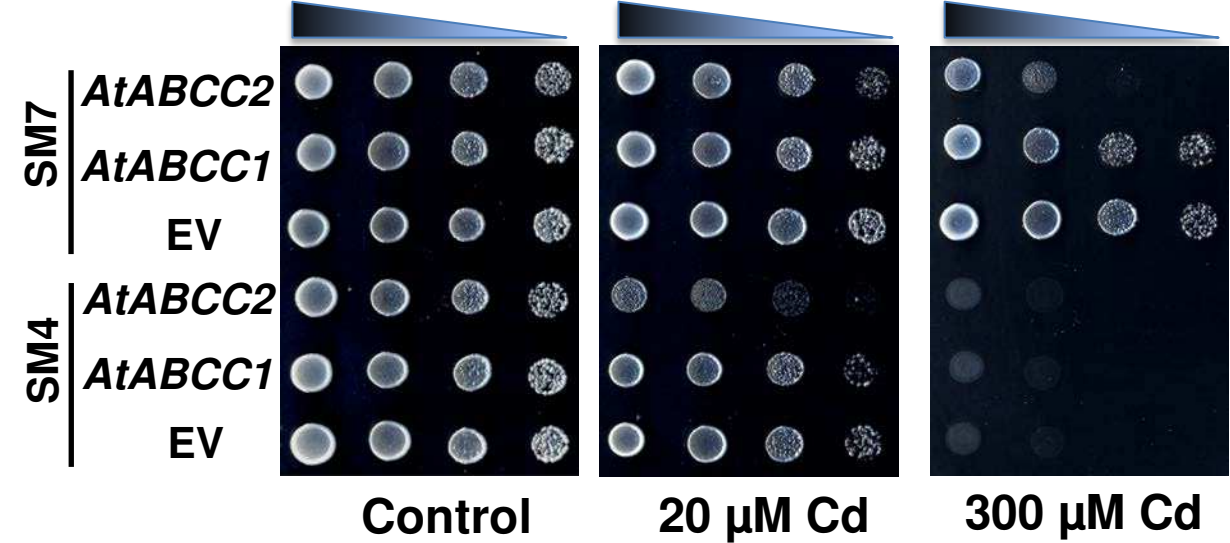


Figure 1.

(a)



(b)

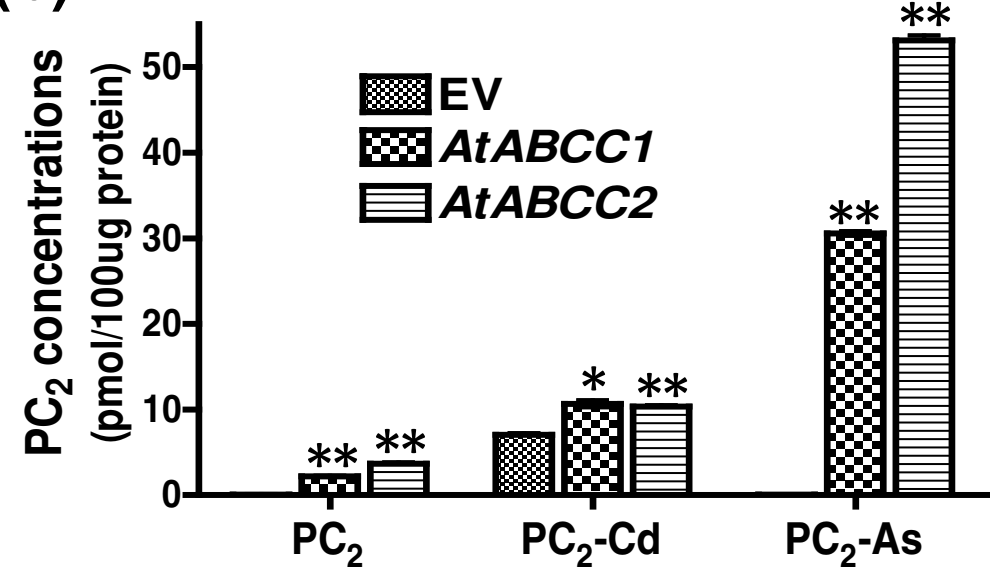


Figure 2.

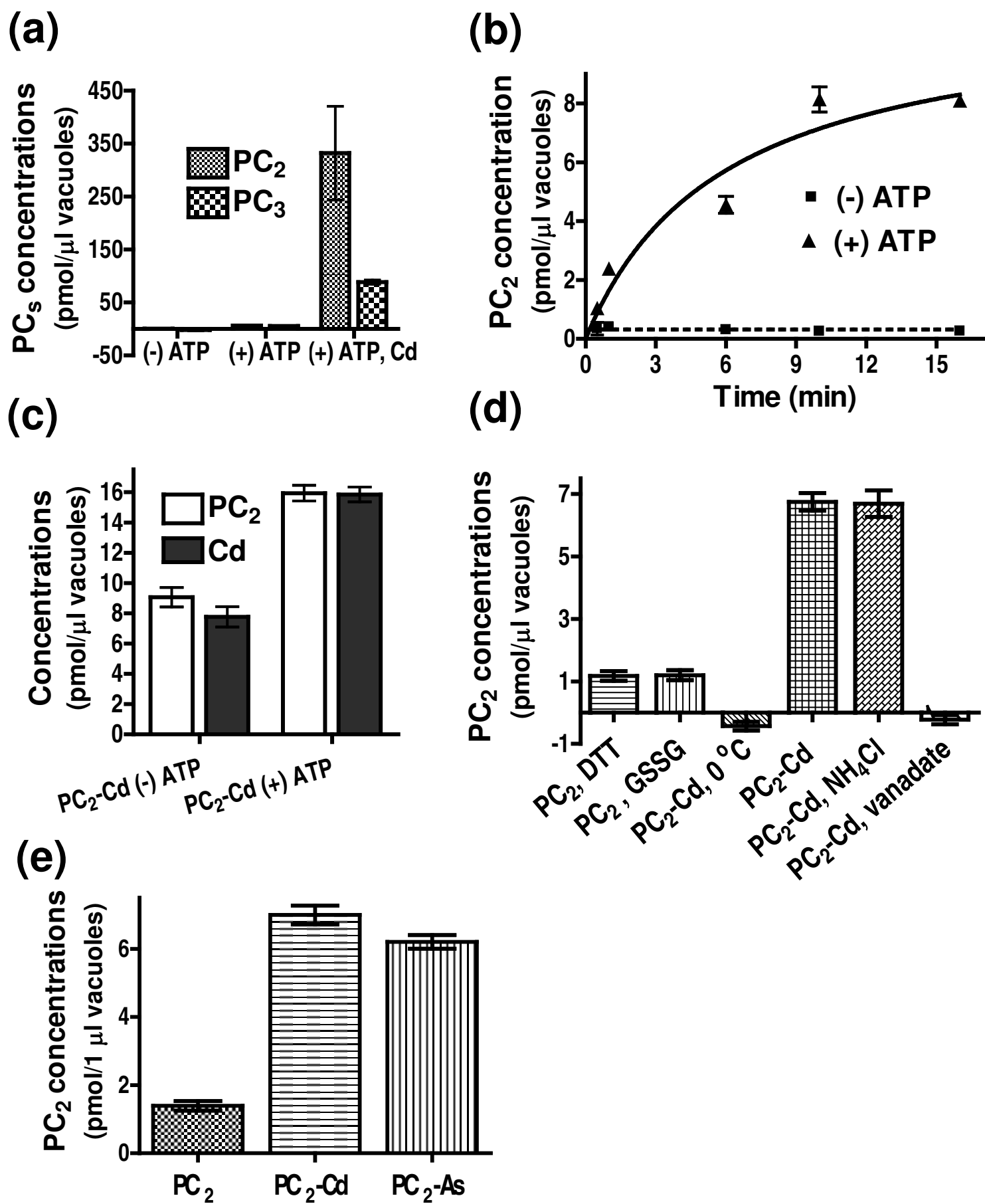


Figure 3.

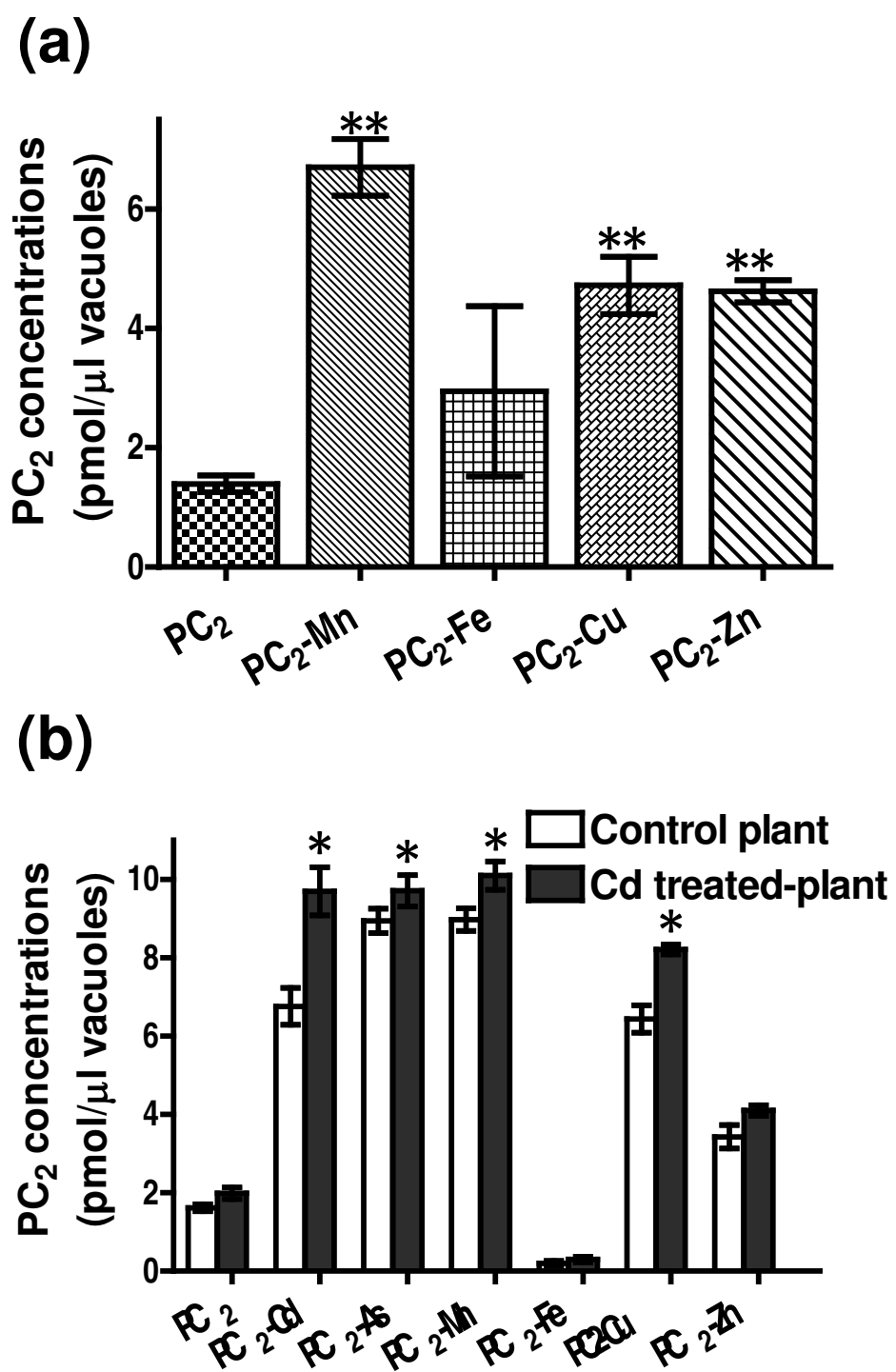


Figure 4.

[illegible]

ABCC domain2

V E D Y S E E N G E A E V D Q T S V K P V E N G N A N N L Q K D G I E T K N
V E E Y S E E N G E A E A D Q T A E Q P V A N G N T N G L Q M D G S D D K K
M E E Q V E E - - D E S K P K D V A K Q T V N G D V - T I A D E G S Q K S Q
M E E Q V E E - - D E S K P K D V A K Q T E N G D V - I I A D E G S Q K S Q
M E E Q M E E K Q D E S Q R Q D D I K H P E N G G S - V I A D G D M Q K S Q
M E E Q T E E K Q D K R K S Q D D I K H T E N G G T - V I A D G G P Q K S Q
M E E Q T E E K Q D E S K S Q D D T K H T E N G A V - V I A D G A P Q K S Q

Figure 5.